

The University of Chicago

A QUANTITATIVE STUDY OF GENIC
EFFECTS ON GUINEA-PIG
COAT COLOR

A DISSERTATION SUBMITTED TO THE FACULTY
OF THE DIVISION OF THE BIOLOGICAL SCIENCES
IN CANDIDACY FOR THE DEGREE OF DOCTOR OF
PHILOSOPHY

DEPARTMENT OF ZOOLOGY

1937

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ELIZABETH SHULL RUSSELL

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A QUANTITATIVE STUDY OF GENIC EFFECTS ON GUINEA-PIG COAT COLORS¹

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INTRODUCTION

THE problem of the manner in which genes produce their effects may be favorably approached in a study of the coat color of mammals, as there is reason to believe that the chain between gene and phenotype is here relatively short. The persistence of color in mutant spots and in transplantation experiments indicates that the pigment is determined locally, and the processes concerned continue throughout life, as hair is constantly being shed and replaced. The guinea-pig is particularly good material for work on pigmentation because of the studies of WRIGHT (1916, 1917, 1925, 1937), who described the effects of its color factors in different combinations and attempted to determine from those the simplest possible scheme for the interactions of the gene products involved. This provides a background for further work, an hypothesis which may be tested.

The main color factors of the guinea-pig fall into three groups. The first group has to do with the restriction of coloration to a piebald pattern on a white ground, and is represented by the pair *S*, *s*, and minor modifiers. The second group has to do with the primary qualitative differentiation of pigment, that between yellow and darker colors. It is represented by *E* (black, sepia, brown, etc.), *e^p* (tortoise), *e* (red, yellow, cream, etc.), and by *A* (agouti), *a^r* (dark agouti), *a* (solid black, etc.). The third group of factors varies the intensity of color in the sepia or yellow series, or both, but not their distribution. This group includes the albino series (*C*, *c^k*, *c^d*, *c^r*, *c^a*), affecting the intensity of both yellows and sepias; *P*, *p* and *B*, *b*, affecting only the sepia series; and *F*, *f* affecting yellows in all combinations but sepias only in combination with *p p*. This paper deals mainly with the third group of factors and somewhat with the qualitative differences between the pigments produced by *E* and *e*.

The colored materials of the hair are melanins, proteins of unknown composition, probably derived from tyrosin, dioxyphenylalanin, or allied

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substances. They are found widely distributed in both vertebrates and invertebrates, although it is highly probable that all melanins are not identical. They are characterized by extreme inactivity and generally by dark color. A description of them and their properties may be found in FÜRTH (1924) and SAMUELY and STRAUSS (1922). The exact manner of formation of these substances is not known, but since the discovery of tyrosinase by BERTRAND (1896), it has been generally accepted that a substrate, or chromogen, and an oxidative enzyme are involved. BLOCH (1917) found that the enzyme in human pigment-forming cells reacts to dioxy-phenylalanin (dopa) and not to tyrosine, and called it dopaoxidase. The results of W. L. RUSSELL (1939) indicate that dopaoxidase is effective in guinea-pig pigmentation also. The studies of RAPER (1928, 1932) have done much to further our knowledge of the possible stages in the reaction leading from tyrosine to melanin. Although knowledge of the concentration of any component of the pigment reaction would be of value in a quantitative study of gene action, one obviously essential step is a determination of the actual quantities of end-product present in various genotypes.

GENERAL METHODS

Quantitative measurements of the melanins in hair are made difficult by their inactivity and the great similarity of their reactions to those of the hair protein, keratin. The possible mechanisms for separating these two components of mouse hair are discussed by EINSELE (1937). He concludes that acid hydrolysis is most suitable, as both keratin and melanin are alkali soluble, but although keratin may be hydrolyzed by sufficiently long treatment with concentrated acid, the melanin is unaffected by this treatment. In view of the distinction made by GORTNER (1912) between acid-soluble and acid-insoluble melanins, it should be noted that EINSELE's statement concerning the insolubility of mouse melanin depends on the fact that the color obtained in the acid hydrolysate is always pale yellow, showing no correlation with the amount of melanin in the hair treated. From this he concludes that no melanin has been dissolved in the acid. By this same reasoning the same conclusion holds for guinea-pig melanin. The acid hydrolysate here is always straw-colored, and no differences in color are found corresponding to the intensity or type of color in the hair, even in the extreme cases, black, red and white. This method may be used for isolating melanin for quantitative comparison of various types on the assumption that if there is any effect of the acid not detected by the color of the hydrolysate, it is approximately the same for all types. EINSELE's method of acid hydrolysis was adopted for the isolation of guinea-pig melanin. However, the lightness and flocculence of the dilute yellows has made their separation from an acid hydrolysate so difficult that results

for these types by this method are not sufficiently accurate, and it has seemed advisable to measure the concentration in yellows by some other method.

As reported by DURHAM (1904), the yellow pigment in guinea-pig hair is much more soluble in alkali than is the sepia. I found the yellow pigment completely and rapidly soluble in cold dilute alkali, while the sepia dissolves very slowly and never becomes completely dissolved by this treatment. This high solubility of yellows in alkali has made possible colorimetric comparisons of the color obtained from the various yellow genotypes, providing the desired substitute for the acid hydrolysis of these types.

EINSELE followed his isolation of melanin by a determination of the percent of the total weight which it formed in each type. He obtained consistent results when the percent weight was two or over, but not below this figure. Unfortunately, the weights of melanin are less in the guinea-pig than in the mouse, and in the pale sepias (*E p p*) and yellows (*e e*), the amount seems to be too low for accurate determinations. A more accurate method useful for these types has been developed in this study. Instead of weighing the isolated melanin, the amount of KMnO_4 necessary to oxidize it has been determined. Permanganate oxidation of melanin was first used by BACH (1908) to measure quantitatively the product obtained by the action of tyrosinase on tyrosine, and was considered by him an accurate indicator.

Thus it has been possible to find three methods for measuring melanin concentration in the hair, although none is suitable for all types:

1. The colorimetric comparison of alkaline solutions of the yellow series.
2. The percent weight of melanin in *P*-sepias as determined by the valuable technique of EINSELE (1937).
3. The permanganate oxidation values of the entire sepia series and the higher members of the yellow series as determined for pigments isolated by the EINSELE technique.

Certain procedures are common to all the methods used in this study, and they may be mentioned here. In all cases, hair was collected from the middle part of the back of two to three week old animals. This hair was removed down to an approximately equal length of base by hair clippers or scissors. Variations in total length of hair combined with variations in depth of color along the hair are an undoubted but unavoidable source of error in these results. The age of two to three weeks seemed advisable as guinea-pigs younger than this did not provide enough hair and the coat color of older animals has been subjected longer to environmental influences. In all cases, two samples from the same animal were used in order to detect gross errors in technique. Unless otherwise stated, no data were

accepted if one of the samples was lost or gave a difference between the two samples for one animal exceeding 25 percent, a very rare occurrence. In all cases, results are classified in two ways, according to genotype and according to a grade of color assigned to each animal. The genotypes were determined by DR. WRIGHT, using as criteria the genotype of the parents, the animal's own color, and, in certain cases, breeding tests. The animals used came from an extensive series of experiments conducted by DR. WRIGHT for the purpose of producing animals of as great a variety of known genotypes with respect to color as possible. The grades were assigned by DR. WRIGHT within one or two days after birth by comparison with a standard set of hair samples, arranged in steps of just perceptible increase in depth of color (WRIGHT 1927). There are thirteen steps in the yellow series, twenty-one in the sepias. The colors change somewhat with age, but there is no appreciable change between birth and three weeks.

COLORIMETRIC METHOD FOR YELLOWS

As stated above, the yellow pigment of guinea-pig hair is soluble in dilute alkali. A measure of the relative amount of pigment in two different genotypes may be obtained by extracting the pigment from equal weights of hair of each type in equal amounts of alkali under identical conditions. The intensity of color in the filtered solutions may then be compared by matching the two solutions in a colorimeter. The color seen in the colorimeter depends upon the intensity of the color in the solution, and upon the height of the column of liquid through which the light passes. In matching a standard color the height of the column of liquid will be shorter for a dark shade than for a pale one. According to Beer's law, the product of height of liquid 1 times intensity of color 1 is equal to the product height 2 times intensity 2 and the ratio of intensities of two samples is, therefore:

$$\frac{\text{intensity 1}}{\text{intensity 2}} = \frac{\text{height 2}}{\text{height 1}}.$$

In my experiments I compared the intensity of color from each hair sample with that of a standard sample from *ee c^d c^d*, yellow of grade seven. It was necessary from time to time to replace the standard because of loss of small amounts in transferring to the colorimeter. When this was necessary, a group of samples from *ee c^d c^d* hairs were made up, and that which agreed most closely with the old standard was taken for the new one. This method of selecting standards might lead, if the originals faded before they were matched, to a gradual reduction in the intensity of the standard. The possibility of a serious shift was avoided by the use of one genotype only, *ee c^d c^d*, those selected always being of grade 7. Six standards were

used in all, the largest number of ratios being determined with the first and fourth in the series. Table 1 shows the ratios obtained with these two standards, classified by grade.

TABLE 1

WRIGHT'S GRADE	STANDARD 1		STANDARD 4		DIFFERENCE 4-1
	MEAN	NO.	MEAN	NO.	
0	.108	2	.107	2	-.001
1			.130	1	
2	.238	6	.299	1	+.061
3	.281	8			
4	.503	8	.634	2	+.137
5	.578	8	.688	4	+.110
6	.714	3	.871	3	+.157
7	.956	16	1.029	9	+.073
8	1.130	1			
9			2.308	1	
10	3.010	8	2.940	6	-.070
11	2.834	4	3.259	5	+.425

Among the eight comparisons which can be made, the ratio is higher in six and lower in two, the average ratio of fourth to first being 1.12. This difference is probably significant (probability .04 by STUDENT'S method), but it is not certain that it indicates a change in the standard as different animals were used in the two series. In any case, it is not serious (less than one-third of a grade), the average increment from one grade to the next being about 38 percent.

TABLE 2

Colorimeter comparison with standard of yellow solutions obtained with different concentrations of alkali.

ANIMAL	CONCENTRATION OF ALKALI (NORMALITY)								
	.25	.5	1.5	2.13	2.75	3.38	4.00	5.25	6.50
1	.895	1.027	.886	.994	.809	.943	.707	1.076	.863
2	.729	.763	.738	.618	.703	.747	.535	.692	.607
3	.808	.885	.950	.888	.875	.978	.925	.938	.905
4	.816	.820	1.158	.910	.831	.780	.865	.902	.840
5	.933	.846	.871		.988	.857	.733	1.008	.813
6	.803	.855	.942	.877	.810	1.013	.918	.857	.847
Mean	.831	.866	.924	(.857)	.836	.886	.781	.912	.813

The effects of various factors on the alkaline solutions were determined. The effect of concentration of alkali was tested, using concentrations from .25/N-6.5/N NaOH. Nine equal samples were taken from each of six ani-

mals and dissolved in equal volumes of NaOH of varying concentration. As can be seen from table 2, no significant changes with concentration appear when these are compared with the standard.

The effect of long boiling was also tested and found to be negligible, providing the original volume was restored before each ratio of boiled to standard was determined. Two 100 cc colored solutions were boiled 3.5 hours with color determinations every ten minutes. The results are given in table 3.

TABLE 3

Effect of long boiling on alkaline solutions of yellow pigment.

MINUTES BOILED	COLORIMETER COMPARISON WITH STANDARD		MEAN
	SOLUTION 1	SOLUTION 2	
10	1.005	.984	.995
20	1.073	1.042	1.058
30	.933	1.003	.968
40	.863	1.060	.961
50	.792	1.040	.916
60	.883	1.045	.964
70	.874	1.073	.974
80	.951	1.109	1.030
90	.869	1.065	.967
100	.999	1.073	1.036
110	.878	1.155	1.017
120	1.007	1.073	1.040
130	.923	1.289	1.106
140	1.071	1.284	1.178
150	.850	1.195	1.023
160	1.082	1.107	1.095
170	1.084	1.185	1.121
180	.924	1.185	1.055
190	.891	1.087	.989
200	.949	1.074	1.012
210	.975	1.165	1.070

The effect of electric light on these alkaline pigment solutions was tested by placing six samples of a homogeneous solution 12 inches from a 60 watt-110 volt lamp for periods varying from 12 to 96 hours and comparing these with a control sample kept in darkness. The ratios are given in table 4.

Similar results were obtained with two other samples at six to 48 hours. As the hair (after collection) and the solutions are kept in darkness except during tests, it is probable that no appreciable fading occurred.

The final method adopted was to wash the hair to be tested in cold ether to remove at least the major part of the fat which might alter the color. From this hair, after drying, two 100 mg samples (to the nearest milligram)

TABLE 4

Effect of light on alkaline solutions of yellow pigment.

HOURS IN LIGHT	COMPARISON TO CONTROL
0	1.010
12	1.005
24	1.000
36	1.005
48	1.013
72	.990
96	1.000

were removed. Each was placed in 10 cc of .5 or .25/N NaOH for five days. The hair was then filtered off and the resulting colored solution compared in a colorimeter (Klett Biometer) with the standard, and the ratio of intensity of tested sample to that of the standard recorded. The two samples from one animal were also checked against each other. Before any comparisons of intensities with different genotypes were made, the ratios were corrected by subtracting the slight amount of color (.120) obtained from white (presumably fat and hydrolyzed keratin). The accuracy of this

TABLE 5

Mean colorimetric values of the yellow genotypes.

GENOTYPE	MEAN WRIGHT'S GRADE	MEAN COLORIMETRIC VALUE	S.E.	NUMBER
<i>eeC-F-</i>	10.33	2.866	$\pm .114$	27
<i>eeck^kF-</i>	6.92	.949	$\pm .055$	10
<i>eeck^dF-</i>	7.00	1.031	$\pm .150$	6
<i>eeck^rF-</i>	4.89	.616	$\pm .048$	13
<i>eeck^aF-</i>	5.00	.489	$\pm .025$	4
<i>eeck^dF-</i>	6.93	1.150	$\pm .082$	23
<i>eeck^dF-</i>	4.16	.593	$\pm .046$	19
<i>eeck^dF-</i>	4.20	.484	$\pm .036$	18
<i>eeck^rF-</i>	0.00	.133	$\pm .017$	2
<i>eeC-ff</i>	7.00	.888	$\pm .053$	7
<i>eeck^dff</i>	2.46	.280	$\pm .031$	10
<i>E-C-ffpp</i>	3.00	.469	$\pm .037$	2

method may be indicated by the variability of the ratios between two samples from the same animal. The ratio of second to first in 152 animals was 1.003 with a standard deviation of $\pm .126$. About one-twentieth of this variability was due to errors in colorimetry. In the determinations of each ratio, three independent readings were made with the colorimeter. The mean ratio of the second of these to the first is .992 with a standard deviation of $\pm .072$. It is necessary to multiply this standard deviation by

$\sqrt{1/6}$ in order to make it comparable to the standard deviation of the ratio of two samples from the same animal (each the average of three colorimetric comparisons), giving .029 or 23.3 percent of .126, making the variance 5.5 percent that of the former ratio. The remaining error in the ratio of two samples from the same animal must be due to variations in the pigment in the hair or in the preparation of the material.

TABLE 6
Mean colorimetric values of Wright's color grades for yellows.

WRIGHT'S GRADE	MEAN COLORIMETRIC VALUE	OBSERVED STANDARD ERROR	STANDARD ERROR CALCULATED FROM COEFF. VARIABILITY	NUMBER
0	0.120	$\pm .016$	$\pm .017$	3
1	0.122	—	$\pm .031$	1
2	0.239	$\pm .026$	$\pm .030$	4
3	0.357	$\pm .030$	$\pm .026$	12
4	0.552	$\pm .027$	$\pm .030$	22
5	0.627	$\pm .046$	$\pm .038$	17
6	0.778	$\pm .049$	$\pm .056$	12
7	1.039	$\pm .047$	$\pm .045$	34
8	1.403	$\pm .197$	$\pm .144$	6
9	2.237	$\pm .117$	$\pm .397$	2
10	2.872	$\pm .156$	$\pm .186$	15
11	3.020	$\pm .199$	$\pm .253$	9
12	2.654	—	$\pm .667$	1

The results obtained with this method are given in table 5 and figure 1 according to genotype and according to WRIGHT's grades in table 6 and figure 2.

It was found that WRIGHT's grades for the C series, as given in this 1927 paper, could best be compared with the colorimetric values by regarding the former as geometric rather than as arithmetic steps. The transformation was accomplished by plotting the logs of the colorimetric values (CV), corrected for the color from white hair, against the grades (G) and finding the best straight line through the points, weighting the means by their generalized standard errors determined from the weighted mean coefficient of variability. This arrangement of the data is shown in figure 2. The formula for the line, as determined by the weighted least square method, is:

$$\text{Log (CV- .120)} = 9.032 + .135 G.$$

The standard error of the slope of this line is $\pm .005$. The results with this transformation of the 1927 grades are given in table 7 and figure 3. This transformation seems justifiable, as it seems probable by the Weber-Fechner law that steps equally spaced to the eye show equal percentage increments rather than absolute increments.

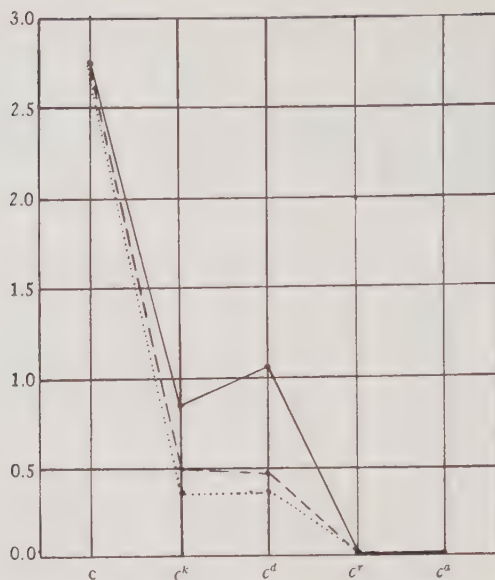


FIGURE 1.—The colorimetric values (ordinate) of various yellow genotypes (abscissa). The solid line shows the values obtained with the homozygote of the allele indicated below the graph; the dash line represents those obtained with its heterozygote with c^r ; and the dotted line those with its heterozygote with c^a .

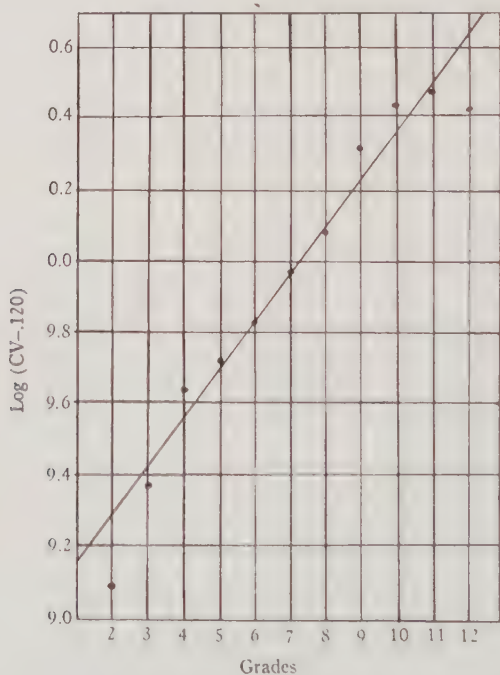


FIGURE 2.—Logarithm of (CV - 120) (ordinate) for each of WRIGHT'S grades for yellow (abscissa).

TABLE 7

Comparison of Wright's grades for yellow genotypes (transformed to a geometric scale) with the mean colorimetric values for the same genotypes.

GENOTYPE	WRIGHT'S GRADE (1927)	TRANSFORMED WRIGHT'S GRADE	CORRECTED COLORI- METRIC MEAN
C-	10.6	2.78	2.75
c ^k c ^k	7.1	.92	.83
c ^k c ^d	7.2	.94	.91
c ^k c ^r	4.6	.42	.50
c ^k c ^a	4.6	.42	.37
c ^d c ^d	7.0	.89	1.03
c ^d c ^r	4.1	.36	.47
c ^d c ^a	4.2	.37	.36

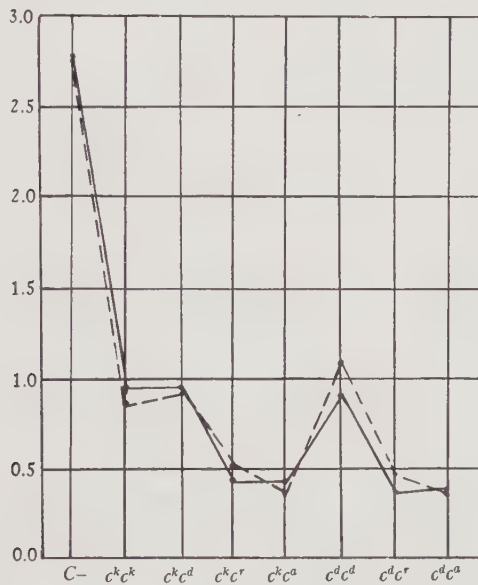


FIGURE 3.—Comparison of the colorimetric values with WRIGHT's grades transformed to a log scale. The solid line connects the transformed WRIGHT's grades, the dash line the corrected colorimetric values.

PERCENT WEIGHT OF MELANIN IN SEPIAS

The method used for this study was the extremely valuable EINSELE technique (1937). This consists of separating the acid-insoluble melanin from the keratin by acid hydrolysis which breaks up the keratin and appears to leave the melanin unmodified. To prepare the hair for this treatment it is necessary to wash it with warm ether for a number of days to remove the fat particles which, upon acid hydrolysis, would form a ring around the sides of the condenser and adsorb a significant part of the

melanin granules. In place of the Jena glass and block-tin condenser used by EINSELE, an all metal apparatus was used in these experiments. This consisted of a rectangular galvanized-tin jar, coated with block-tin to prevent the entrance of impurities into the ether. Inside this cylinder the hair rested in nichrome baskets on a set of ten nichrome (ether resistant) screen trays, supported on a block-tin coated galvanized-tin rack. A five centimeter layer of ether was maintained in the bottom of this jar. The top of the cylinder was an inverted truncated pyramidal chamber, through which cold water ran continuously, with a drip plate on its lower surface the same size as the nichrome trays and directly above them. The ether vapor recondensed on this plate, and thus fell directly onto the hair. Ether leakage was prevented by a Bruhl wax seal and by equalization of the pressure inside the ether bath with that of the atmosphere by inserting an ordinary open condenser into the top of the bath, allowing for escape of air but preventing escape of ether. Heat was applied to the bath by two 100 watt electric bulbs enclosed in a separate chamber fitting beneath the main jar.

The procedure followed was to wash the hair (about .750-1.000 grams from each animal) in 0.1 percent sodium lauryl sulphate for one hour. This high molal salt of a fatty acid is sold commercially as "Dreft," and has the saponifying qualities of soap, but is neutral in reaction (a feature necessary for washing the alkali-soluble yellows), and can be used cold, which is further guarantee of retention of the yellow pigment. (I am grateful to DR. D. G. STEELE for this suggestion concerning technique.) The Dreft was decanted and followed by four washes with hot distilled water. Each sample was transferred to a nichrome basket and dried in an oven at 90-100°C. The dried cooled hair was placed in the ether bath, two samples to a tray, and washed with ether. By daily weighing tests, it was found that all of the fat was removed in four days, so five days was accepted as a safe standard time for washing with ether. On removal from the bath, each sample was divided into two samples and dried. The weight of the dry hair was determined (mean weight .377 grams) and the hair was transferred to an all-glass reflux condenser and boiled for one hour with 50 cc 6 N HCl. At the end of this hydrolysis, the hair was completely broken up and the melanin found as free granules. Addition of 25 cc of 95 percent alcohol to the hydrolysate was found to facilitate separation of the melanin granules from the liquid. (I am grateful to DR. STEELE for this suggestion also.) This separation was accomplished by centrifuging, ten minutes at 3600 rpm. being sufficient for practically all types. The liquid was decanted, replaced by distilled H₂O, and the melanin again centrifuged out. This was done four times, and tests of the fourth wash were always negative for chloride, indicating that all of the acid, and consequently all of the dis-

solved keratin, had been removed. The precipitate was then transferred to weighing bottles, dried, and weighed. The final data recorded were the mean percentage weights of melanin in the two parts of each hair sample. The two samples from each animal serve as a check on gross errors. This method was found to be satisfactory only for *P*-sepias, as *p*-sepias and yellows have smaller weights of pigment, and the error is so great as to make the results of little value.

This determination was used for two *P*-sepia genotypes, in order that the results might be compared with, and act as a check upon, the numbers obtained for these genotypes by the permanganate method. The results are given in table 8. The percent of acid-removable substances in white was also determined by boiling white hair samples with black of known melanin content and determining the increment due to substances adsorbed from the whites. This value, the percent weight of melanoidins and other impurities resulting from acid hydrolysis of hair, was subtracted from each mean to give the true percent content of melanin. By this method *c^d c^a E F P* has 47.9 percent as much pigment as *C-E F P*.

TABLE 8
Percent weight of melanin in two sepia genotypes.

GENOTYPE	MEAN	S.E.	S.D.	NUMBER	TRUE MEAN
<i>C-E-F-P-</i>	3.34	± .21	± .67	10	2.86
<i>c^d c^a E-F-P-</i>	1.85	± .13	± .42	10	1.37
White	0.48	± .09	± .27	9	0.00

THE PERMANGANATE METHOD

A smaller difference between the two samples from one animal and among all the animals of the same genotype was found with a modification of the method given in the foregoing section. In this method the isolated melanin, and the small amount of distilled water left after the fourth decantation after washing, were transferred to a 150 cc beaker, acidified with 2 cc concentrated H_2SO_4 , and heated to the boiling point. The hot mixture was titrated against .005-.01 *N* $KMnO_4$ until the addition of .5 cc left a pink color after one minute of stirring. With samples of high melanin content the amount added was increased to 1 cc to compensate for volume differences. BACH (1908) states that it is sufficient that the black melanin mixture be titrated with permanganate just to decolorization in order to obtain consistent results. I attempted to use this method, but could not decide exactly when the melanin reached "decolorization." It continued to reduce potassium permanganate rapidly for a time after losing all color, and samples left for several hours always lost their pink color no matter how great an excess of permanganate had been added. The explanation

of this phenomenon is not known. Since BACH apparently titrated his samples only until they were decolorized, he did not encounter this difficulty. The decolorization point may have been easier to determine in his material, as the melanin was undoubtedly purer and in finer particles, being made artificially by the action of tyrosinase on tyrosine in solution. As my material was oxidized more, rather than less, than BACH's, it seems probable that the entire amount of pigment is measured by the permanganate oxidation at all points in the scale of intensity of color.

The timed end-point makes this method liable to considerable subjective error, but every attempt has been made to keep this at a minimum. The policy followed was to keep the operator ignorant of the genotype being tested and the result obtained for a second sample from the same animal until titration was completed, to keep lighting and temperature as constant as possible, and to seek corroboration of the end-point whenever feasible. As considerable calculation is necessary before the final value is obtained, there is little danger of trying to match samples.

Potassium permanganate can not be made up to a desired normality, but its normality can be accurately determined. The policy was, therefore, to make up KMnO_4 somewhere near .005-.01/N, and determine the normality with $\text{Na}_2\text{C}_2\text{O}_4$. Comparable figures for different KMnO_4 solutions were obtained by expressing the final results in a form called for convenience the permanganate number:

$$(\text{PN}) = \frac{\text{cc KMnO}_4 \times \text{N KMnO}_4 \times 100}{\text{grams hair}}.$$

Tests were made with .016/N and .038/N KMnO_4 and the results obtained this way were much more different from those obtained within the limits .005-.01/N KMnO_4 than any of those were from each other. This seems to indicate an effect of concentration of permanganate, which should be further investigated. The permanganate numbers with the higher normality permanganate were too high in all genotypes tested. Analyses of the data show no significant effect of this factor in the range from .005-.01/N, and the data obtained with higher concentrations were discarded.

A second difference from the method described earlier was the addition of 100 mg of black of known permanganate value to each sample before boiling. The addition of black was found by EINSELE to be helpful in dealing with light pigments which become adsorbed on the melanin granules of the black and thus centrifuge out of the solution easily. The samples added were obtained by combining all of the hair from the backs of a number of black guinea-pigs, and working them into a homogeneous mixture. After washing in the ether bath, this hair was divided into 100 mg samples (about 200 samples from one batch of hair) and determinations made of

TABLE 9

Coefficients of variability obtained with the permanganate and percent weight of melanin methods.

GENOTYPE	COEFF. VAR. (PERMANGANATE NUMBER)	COEFF. VAR. (% WEIGHT MELANIN)
<i>C-E-F-P-</i>	10.5	19.2
<i>c^d c^a E-F-P-</i>	18.5	21.6
White	16.8	52.1

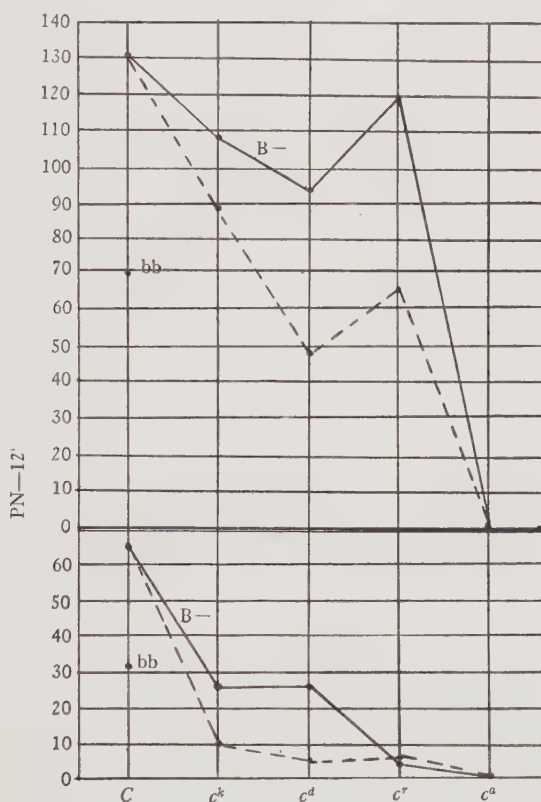


FIGURE 4.—The corrected mean permanganate number for various sepia and brown genotypes. The upper figure shows the darker sepias and browns, the lower the pale sepias and browns. The solid line shows the values obtained with the homozygote of the allele indicated below the graph, while the dashed line indicates those obtained with its heterozygote with *c^a*.

the permanganate numbers of ten of these, chosen at random. The mean amount of KMnO_4 , in terms of mols, used up by these samples was then subtracted from the total permanganate used by each sample. The amount which this addition of black hair adds to the variability of the final permanganate values may be estimated from the standard deviations of the mean permanganate numbers of the blacks for combination. Two series

TABLE 10

Permanganate numbers observed for each of the genotypes tested.

C SE- RIES	PERMANGANATE NUMBERS																NO.	MEAN	S.E. *
	0- 9	10- 19	20- 29	30- 39	40- 49	50- 59	60- 69	70- 79	80- 89	90- 99	100- 109	110- 119	120- 129	130- 139	140- 149	150- 159			
<i>E- F- P- B-, darker sepias</i>																			
C-											1				3	2	1	7 143 ± 9	
<i>c^k c^k</i>										1			1		1			3 120 ± 12	
<i>c^k c^r</i>																	1	1 166 ± 26	
<i>c^k c^a</i>											1							1 100 ± 17	
<i>c^d c^d</i>											2							2 106 ± 13	
<i>c^d c^r</i>									1	1		2	2		1		1	8 121 ± 7	
<i>c^d c^a</i>					1	3	3			1								8 60 ± 4	
<i>c^r c^r</i>												1	1				1	3 131 ± 12	
<i>c^r c^a</i>						1	1	1	2	1								6 77 ± 6	
<i>E- F- p p B-, pale sepias</i>																			
C-									1									1 76 ± 14	
<i>c^k c^k</i>				3	3	1		1										8 37 ± 3	
<i>c^k c^d</i>				1														1 23 ± 7	
<i>c^k c^r</i>				1														1 29 ± 7	
<i>c^k c^a</i>			3	3														6 21 ± 3	
<i>c^d c^d</i>						2												2 37 ± 6	
<i>c^d c^r</i>			2	4	1													7 21 ± 2	
<i>c^d c^a</i>				3	1													4 17 ± 3	
<i>c^r c^r</i>				4	1													5 16 ± 3	
<i>c^r c^a</i>	1			2														3 18 ± 3	
<i>E- F- P- b b, darker browns</i>																			
C-								2	2	1								5 81 ± 7	
<i>c^k c^a</i>									1									1 83 ± 15	
<i>E- F- p p b b, pale browns</i>																			
C-					1	1												2 43 ± 7	
<i>e e F-, yellows</i>																			
C-				1	4	2												7 38 ± 3	
<i>c^k c^k</i>		1																1 18 ± 5	
<i>c^k c^r</i>		2																2 17 ± 3	
<i>c^k c^a</i>		1																1 18 ± 5	
<i>c^d c^d</i>			2	3														5 21 ± 2	
<i>c^d c^r</i>	1		3	3														7 17 ± 2	
<i>c^d c^a</i>		1		2														3 21 ± 3	
<i>e e f f, pale yellows</i>																			
C-			3	1														4 18 ± 3	
<i>c^d c^d</i>	1		3															4 12 ± 2	

* (The standard errors were determined on the basis of a constant coefficient of variability, the weighted mean coefficient of variability of all types involved. In taking all of the coefficients of variability, allowance was made for the variability of the 100 mg of black hair added to each sample, by adding to the mean permanganate number for each type an amount equal to the product of the mean fraction of the total weight of the sample constituted by this black, and the weighted mean permanganate number of this black. The actual amount added was 26.)

were used, the first having a mean value of 108 with a standard deviation of 8, the second a mean value of 146 with a standard deviation of 4. Allowance for this factor was made in the determination of the standard errors

TABLE II
Permanganate numbers observed for each of Wright's grades.

WRIGHT'S GRADES	0-	10-	20-	30-	40-	50-	60-	70-	80-	90-	100-	110-	120-	130-	140-	150-	160-	NO.	MEAN	S.E.*
	9	19	29	39	49	59	69	79	89	99	109	119	129	139	149	159	169			
Sepias																				
0-1	7	5	3															15	12	± 1
2		8	1															9	15	± 2
3		2	4															6	19	± 2
4			2	1														3	27	± 4
5		2	3															5	21	± 3
6			2	1														3	32	± 4
7				1	1													2	39	± 5
8			2	1														3	32	± 4
9				1			1											2	50	± 6
10																				
11																				
12					1			1										2	62	± 7
13						2	1											3	56	± 6
14							3											2	64	± 6
15						1	1	1	2	1								6	75	± 5
16										1								1	99	± 15
17									1		1							2	96	± 10
18											1							1	103	± 15
19										1	1	2	1				1	6	120	± 7
20										1	1	1	1		1			5	115	± 7
21										1	1	1		2		4	2	9	144	± 7
Yellows																				
0-1	7	5	3															15	12	± 1
2	1	2																3	11	± 3
3	1	1	1															3	14	± 3
4		3	2															5	19	± 3
5		1	1															2	20	± 4
6		2																2	17	± 5
7		6	5															11	20	± 2
8				1														1	39	± 9
9			1	1	1													3	37	± 5
10				2														2	36	± 6
11																				
12					1													1	42	± 9

* (Standard errors were determined as in Table 10.)

of all types tested. This method not only made possible tests for pale sepias and yellows which otherwise would not have precipitated well, but also gave accurate tests of white which indicate the part of each permanganate titration due to melanoidins (darkish materials of unknown composition which appear in small concentrations with acid hydrolysis of keratin) and other impurities. This method was more accurate than the weighing method for the lower concentrations, as can be seen from the coefficients of variability of the means for the genotypes tested by both methods, as given in table 9.

Even with this method only the higher genotypes of the pale sepia and yellow series can be taken seriously, but they at least give the approximate location of these types on the total scale. The results obtained with this method are given in table 10 and figure 4 according to genotype, and according to WRIGHT'S grades in table 11 and figure 5. In comparing the

permanganate numbers to WRIGHT's grades the same procedure has been followed as with the colorimetric data. That is, WRIGHT's grades are considered as geometric increments and the permanganate numbers as arithmetic. The exact relationship between the two scales was found by plotting the log of the mean permanganate number for each of WRIGHT's grades against the grades (see figure 5). The permanganate numbers were corrected for adsorbed impurities by subtracting 12 (the mean permanganate number of all samples of grades 0 to 1 in either series, these having only

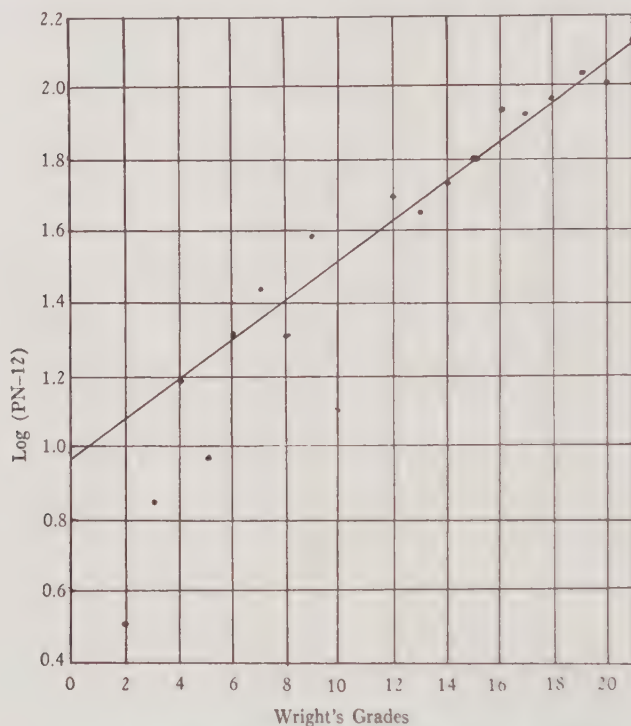


FIGURE 5.—The log of the mean permanganate number for each of WRIGHT's color grades for sepia, plotted against the grades, with the calculated best straight line through all grades from 2-21.

the faintest tinge of color) from all the means. The formula for the best straight line through all grades from 2-21, as determined by the least squares method, with values weighted by their standard errors, is:

$$\text{Log (PN - 12)} = .953 + .056 G.$$

The standard error of the slope of this line is $\pm .0025$. A comparison of WRIGHT's grades for sepia genotypes, transformed according to this formula, with the mean permanganate numbers observed for each genotype is given in table 12 and figure 6. Good agreement is found in all cases ex-

TABLE 12

Comparison of Wright's grades for sepia genotypes (transformed to a geometric scale) with the mean permanganate numbers for the same genotypes.

C SERIES	WRIGHT'S GRADE (1927)	TRANSFORMED WRIGHT'S GRADE	CORRECTED MEAN PERM. NUMBER
Sepias			
C-	21.00	135	131
c ^k c ^k	20.10	120	108
c ^k c ^r	20.46	126	154
c ^k c ^a	18.53	98	88
c ^d c ^d	16.88	79	94
c ^d c ^r	19.09	105	109
c ^d c ^a	14.02	55	48
c ^r c ^r	20.09	120	119
c ^r c ^a	15.49	66	65
c ^a c ^a	0.00	0	0
Browns			
C-	15.63	67	69
c ^k c ^a	13.60	52	71

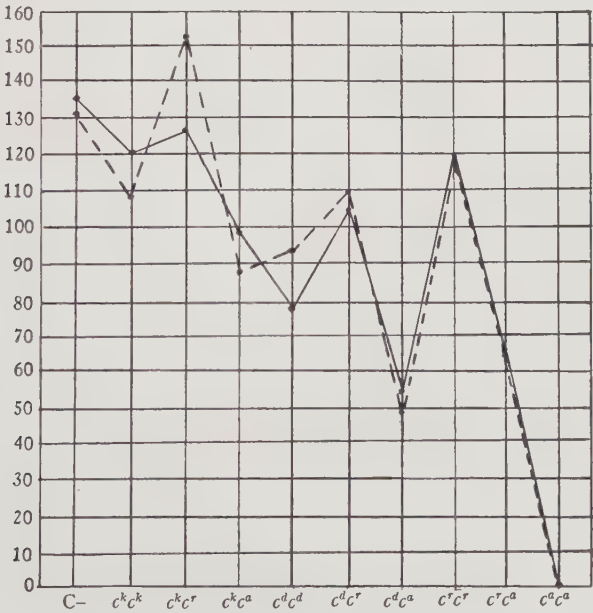


FIGURE 6.—A comparison of WRIGHT'S grades, transformed to a log scale, for each darker sepia genotype (solid line) with the mean corrected permanganate number (dash line).

cept $c^k c^r$, where the observed permanganate number, based on a single animal, has a standard error of ± 26 , sufficient to account for the aberration.

Comparisons between the two samples from the same animal gave a mean difference of 8 ± 1 (11 in darker sepias, 9 in browns, 5 in pale sepias, and 6 in yellows), which gives some idea of the experimental error. The relative undependability of the lower grades of pale sepias is brought out by these figures and also shows up clearly in table 10.

A further experiment with this method was the comparison of sepia litter mates of genotypes $c^d c^a$ and $c^r c^a$, in an attempt to determine the reality of the darker color in $c^r c^a$, which appears in WRIGHT's grades and in my data, by eliminating as far as possible strain differences which might produce this result. These results are given in table 13. They include data

TABLE 13
Litter mate comparisons of permanganate number of $c^d c^a$ and $c^r c^a$ sepias.

LITTER	AGE	MEAN P.N. $c^d c^a$	MEAN P.N. $c^r c^a$	$c^r c^a - c^d c^a$
1	2-3 weeks	56	83	+27
2	2-3 weeks	62	79	+17
3	2-3 weeks	47	82	+35
4	11 months	79 71	86	+11
		75		
5	2 weeks	99	92 83	-11
			88	
Mean Difference				+16 ±8

on older animals as well as the two-three weeks old guinea-pigs used in the regular experiments. The significance of the difference between the two genotypes can be tested by STUDENT's method, using averages where there is more than one of a genotype in a litter. The result ($t = 2.0$, $n = 4$) yields a probability of .12 of a greater difference by accidents of sampling. The difference can thus be considered merely suggestive of a greater amount of pigment in $c^r c^a$ than in $c^d c^a$.

It is important to test the agreement between the permanganate method and the percent weight of melanin method. There is 36.6 percent as much melanin in $c^d c^a$ *E F P* as in $c^r c^a$ *E F P* by the permanganate method, 47.9 percent by the percent weight method. The difference between these ratios is 1.6 times its standard error, and is thus not significant.

$$\left(E \text{ ratio} - E_{x,y} \frac{\bar{X}}{\bar{Y}} \sqrt{\left(\frac{E_x}{\bar{X}} \right)^2 + \left(\frac{E_y}{\bar{Y}} \right)^2}, \quad E_{r_1-r_2} = \sqrt{(E_{r_1})^2 + (E_{r_2})^2} \right)$$

$$r_{r_1-r_2} = .113, \quad E_{r_1} = .040, \quad E_{r_2} = .057, \quad E_{r_1-r_2} = .070.$$

INTERPRETATION

C series

As may be seen from figure 6, the permanganate numbers agree well with WRIGHT's color grades for the darker sepias. It is especially noteworthy that all c^r combinations are higher than the corresponding c^d combinations in both, a reversal of the order in the yellow series.

The chief conclusions for the darker sepias aside from those already made by WRIGHT concern dominance relations of the lower alleles of the *C* series. It is seen that c^r has little or no dominance over c^a , that is, $c^r c^a$ has approximately one-half as much pigment as $c^r c^r$. Correcting the means for adsorbed impurities by subtracting 12 (the value for white), we get $c^r c^r = 119$, $c^r c^a = 65$. There is therefore 55 percent as much pigment in $c^r c^a$ as in $c^r c^r$. With the uncertainty of the means, it is impossible to tell whether or not there is a slight dominance of c^r , but it is quite likely that there is none. Similarly, there is 51 percent as much pigment in $c^d c^a$ as in $c^d c^d$. In WRIGHT's transformed grades the percentages are 55 percent and 70 percent respectively. There is little data for the c^k combinations. The observed percentage in $c^k c^a$, as compared with $c^k c^k$ (81 percent), is, however, practically the same as that in WRIGHT's transformed grades (82 percent). Thus c^d and c^r show approximate intermediacy of the heterozygote with c^a , while c^k shows considerable dominance over c^a . *C* in the sepia series has been found by WRIGHT's grades to be completely dominant in all combinations, and there is nothing in my data to alter this conclusion. WRIGHT suggests (1934) that "under the theory of control of rate" of a reaction in the series of processes between gene and character "by the limiting factor, there is exact intermediacy of the heterozygote (up to a certain level), then a range of increasing dominance of the higher allelomorph until at a certain level complete dominance is attained." This agrees well with the situation in the *C*-series acting on sepia pigment, except that c^k (in the sepia series a higher allele than c^d and c^r because of its partial dominance) homozygotes should be as high as *C*-. It is interesting to note that DUNN and EINSELE (1938) find a similar condition in mice, where the highest allele, *C*, is completely dominant in the black series and a lower allele, c^{ch} , shows no dominance (percent weight melanin in *C*- = 7, in $c^{ch} c^{ch}$ = 5, and in $c^{ch} c^a$ = 2.5.)

A striking contrast between the results for sepias and yellows is in the amount of difference between the values for the dominant *C* factor and for the lower alleles. In sepias, the corrected value in relation to 100 percent in *C E F P* is 82 percent in $c^k c^k E F P$, and 72 percent in $c^d c^d E F P$. In yellows the corresponding figures are 23 percent of *C e e* for $c^k c^k e e$ and 35 percent for $c^d c^d e e$ by the permanganate method, and 30 percent

and 38 percent by the colorimetric determinations which are undoubtedly more accurate. For the dominance effects of the lower alleles in yellows, only the colorimetric data seem worth considering. It must be remembered that both c^r and c^a are inactive in the yellow series. In these, $c^k c^r$ has 59 percent and $c^k c^a$ 44 percent as much color as $c^k c^k$. Similarly, $c^d c^r$ has 47 percent, and $c^d c^a$ 35 percent, as much as $c^d c^d$. C has been shown by WRIGHT to be completely dominant. These results suggest that in the yellow series as well as in the sepias, the gene product in relation to its substrate is in defect in the lower alleles, c^k and c^d , but in excess in C . There is no partially dominant allele here, but the wide gap between $c^{kd} c^{kd}$ and C - may account for this. The approximate identity of effect of c^k and c^d in effects on yellows, shown in WRIGHT's grades, is also supported here. The uncorrected mean for $c^k c^k$ by the colorimetric method is $.940 \pm .055$, for $c^k c^d$ $1.031 \pm .151$, for $c^d c^d$ $1.150 \pm .182$, and all three means are within the limits of error of the mean of the combined population, $1.084 \pm .054$.

The results for the pale sepias are not as clear-cut as the others, considerable uncertainty resulting from lack of numbers in certain genotypes. The most striking feature is the great increase of C over $c^k c^k$, quite different from the darker sepias. However, the corrected value of 64 for C - depends on only one animal. The amount of pigment in $c^k c^k$ is 43 percent of that in C -, close to the ratio in yellows rather than to that in the darker sepias. The heterozygotes of c^k and c^d with c^a show intermediacy, as in the other series. The amount of pigment in $c^k c^a$ is 36 percent of that in $c^k c^k$, that in $c^d c^a$ is 20 percent of that in $c^d c^d$. The extremely high value of $c^{kd} c^{kd}$ is something which must be rechecked, as it is far out of proportion to the color.

The simplest interpretation for the C series of alleles, as far as they have been tested, seems to be that the dominance of C is brought about by the excess of its product somewhere in the chain of processes between gene and character, while the smaller product of the lower alleles is in defect in relation to the substrate in this same reaction. Also in some way the lower members of the albino series reduce yellow much more drastically than black, this effect being manifested not only in the difference in threshold (no yellow but much sepia in $c^r c^r$) but also in the relation of $c^{kd} c^{kd}$ to C -.

The E, e genes

The most striking difference observed between pigments produced with E and e is their solubility in NaOH. The sepia pigments, both in the hair and isolated, go into solution in cold alkali very slowly, and have never in my experimentation become anywhere near completely dissolved. EINSELE (1937) and DANIEL (1938) have been able to dissolve isolated mouse melanin by boiling in N/10 KOH for 3-5 hours, and the same is possible

for guinea-pig melanin, although the experiment had not been performed at the time of this work. The yellow melanin, on the other hand, dissolves completely and rapidly in cold dilute alkali. In fact, the use of soap had to be discontinued as its alkalinity dissolved out part of the pigment. This difference in solubility of melanins was found for the guinea-pig by DURHAM (1904). GORTNER (1911) describes alkali soluble and insoluble melanins from various sources, but makes no statement as to the relation of this solubility difference to color. SALLER and MAROSKE (1933) found this same difference in solubility in human hair, as did GOERNITZ (1923) in birds and DANNEEL (1936) in rabbits. Many authors, such as BOGART and IBSEN (1937), have maintained that this red pigment is present in the hair entirely in a dissolved state, but this cannot be true in the guinea-pig. In our experiments it has been possible to extract an appreciable amount of solid pigment from yellow hair by acid hydrolysis, and the amount of this pigment, as determined by the permanganate numbers, varies with the genotype in the same general way as that extracted in alkali. There seem to me to be two possible explanations for this greater solubility of the yellow pigment in alkali. The first is that the sepia pigment, although identical with the yellow chemically, is present in such large granules, so densely packed, that it cannot be attacked readily by the alkali. The other explanation of this phenomenon is a chemical difference between the two types of pigment. There is nothing in my experimentation which throws light on the nature of this difference. Working with mouse melanin, DUNN and EINSELE (1938) have found graded variations in the size of pigment granules from various genotypes in the black series, and DANIEL (1938) has found no significant differences in the chemical constitution of mouse melanin from various genotypes in a spectrophotometric study of the dissolved isolated pigment. However, it must be remembered that these papers deal entirely with the black and brown series, not with the difference between these and yellows, where the situation may be quite different. In the rabbit DANNEEL has found distinct differences in the histological arrangement of yellow and black pigment in the hair.

The F, f, P, p, and B, b genes

The effect of the *F, f* pair of genes on yellows was tested for two genotypes only, *C-eeff* and *c^dc^deeff*. It is interesting to compare the reduction of yellow by combinations in the albino series in the presence of *ff* with that by the same combinations in the presence of *F-*. *c^dc^d* has 37.5 percent as much pigment as *C-* with *F-*, but only 19 percent as much with *ff*. It may be noted that the reduction is even greater in *c^dc^a*, as *c^dc^aF-* has considerable pigment, while *c^dc^aff* is pure white (except for occasional traces of cream on the nose, cf. WRIGHT 1927). In combination

with $E p p, ff$ causes the removal of all pale sepia pigment. One genotype only, $E- C- ff p p$, was tested colorimetrically. The color and the ratio to $ee c^d c^d$ were close to those of a grade 4 cream, the ratio being $.469 \pm .038$.

The effect of $p p$ aside from that in conjunction with ff is to make sepia paler, but whether this is entirely due to a decrease in quantity of the same pigment, or a change in the type of pigment, can not be stated here.

Whatever change $p p$ produces in sepia pigment, the small amount of data collected here indicate that it has the same effect on browns; that is, $C- E- p p B-$ has 49 percent as much pigment as $C- E- P- B-$, and $C- E- p p b b$ has 45 percent as much as $C- E- P- b b$.

SUMMARY

1. A method has been developed for colorimetric comparison of alkaline solutions of yellow pigment of guinea-pig hair. Tests have been made of all available genotypes.

2. The percent of the total weight of the hair formed by the melanin has been determined in two of the darker sepia genotypes by the EINSELE technique.

3. A method for determining the melanin content of sepias, pale sepias, and, to a limited extent, yellows, has been developed by a modification of the EINSELE technique, in which the isolated pigment is oxidized with potassium permanganate. Tests of all available genotypes have been made with this method.

4. The dominance relations of C, c^k, c^d, c^r, c^a indicate that in sepias, pale sepias, and yellows, the homozygotes of the lower active alleles produce approximately twice as much pigment as their heterozygotes with the inactive gene or genes, while the highest allele appears to be completely dominant. This suggests that in some reaction between gene and character the gene product is in defect in relation to the substrate with the lower alleles and in excess with the highest member of the albino series.

5. The lower alleles of the C series reduce the amount of pigment much more drastically in yellows and pale sepias than in darker sepias.

6. Measurements of the effects of the P, f, P, p , and B, b series are given, with some suggestions as to the nature of the action of these genes.

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